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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The purpose of our research was the generation of relevant biological assay systems in which Ras-independent effects of neurofibromin on cellular proliferation could be assessed. Our primary efforts have been aimed at overcoming longstanding difficulties of manipulating normal and mutant forms of neurofibromin in mammalian cells. We initially focused on development of tightly controlled expression systems using vector- and retroviral-based ecdysone systems and a tetracycline-regulated amplicon system in established NIH3T3 murine fibroblasts and neurofibromin-deficient primary mouse embryo fibroblasts (MEFs). Our progress has been limited by technical difficulties with all three systems that prevented establishment of reliably inducible exogenous neurofibromin expression in either cell type. We have utilized a recently developed modified tetracycline-regulated amplicon system that affords tighter control of exogenous gene expression. Our preliminary analyses in NIH3T3 fibroblasts, neurofibromin-deficient MEFs, and human Schwann cells derived from NF1-associated tumors show improved control of tetracycline-inducible gene expression and suggest that this amplicon system will be a valuable tool in analysis of neurofibromin function in a broad spectrum of cell types, including those that are pathologically relevant to the NF1 phenotype. We expect that further studies utilizing the amplicon system will help to clarify the complex role of neurofibromin in cellular growth control.

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Table of Contents

Introduction

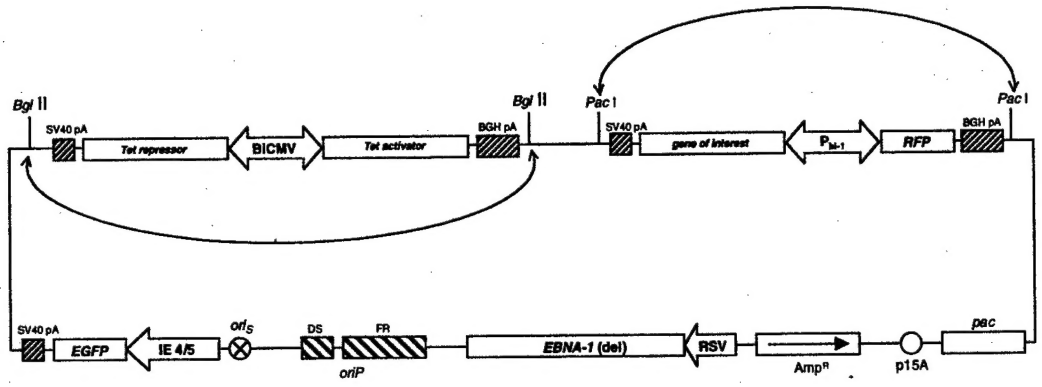
Neurofibromatosis type 1 (NF1) is a common human autosomal dominant disorder characterized by a complex disease phenotype that includes the development of both benign and malignant tumors of the nervous system (1). The *NF1* gene encodes a 250 kD tumor suppressor protein, designated neurofibromin, with clear homology to Ras GTPase activating proteins (GAPs) (2, 3). The ability of neurofibromin to negatively regulate Ras through stimulation of GTP hydrolysis is consistent with its tumor suppressor function. Considerable evidence suggests that neurofibromin has important regulatory functions distinct from its RasGAP activity suggesting its role in cellular signal transduction and growth control is complex. Studies of the Drosophila *Nf1* gene have determined that the primary function of neurofibromin in flies is the regulation of cAMP-mediated signaling (4). The activity of neurofibromin in this process can be dissociated from its established function in Ras regulation (5). A RasGAP-independent function of neurofibromin is further suggested by the observation that 2-3 fold overexpression of neurofibromin in NIH3T3 cells blocked their proliferation (6). This growth-inhibitory effect was not dependent on the ability of neurofibromin to negatively regulate Ras GTPase activity. One additional indication of Ras-independent functions of neurofibromin is derived from comprehensive mutational analyses of the *NF1* gene in affected patients. These analyses suggest that defects in the GAP-related domain (GRD) are not solely responsible for the disease phenotype and the region immediately amino-terminal to the GRD may represent a distinct functional domain of neurofibromin (7, 8). The purpose of our research was to test our hypothesis that neurofibromin has important functional domains distinct from the GRD using a cell proliferation assay in which we could test the effects of various mutant forms of neurofibromin on cell growth in both established and primary mouse fibroblasts. Through these analyses, we expected to define critical domains of neurofibromin and clarify its role in the control of cellular proliferation.

Body

Task 1. Generation of stable NIH3T3/EcR cell lines

The primary goal of task 1 was the development of stable NIH3T3 lines that allow controlled expression of exogenous neurofibromin. The anti-proliferative effect of neurofibromin overexpression in NIH3T3 fibroblasts provides a basis for the development of a cell culture system to study this property. The additional observation that this function of neurofibromin is not dependent on its RasGAP activity makes this assay system ideal for identifying additional functional domains of neurofibromin. We initially utilized a tightly regulatable vector-based ecdysone-inducible expression system (Invitrogen) to generate cell lines in which the anti-proliferative effects of neurofibromin overexpression could be first confirmed and then further studied using mutant forms of the protein (task 2). Our midterm report described our analysis of over 140 NIH3T3 cell lines that carried the ecdysone-responsive transcriptional activator (EcR) plasmid. None of these lines were able to reproducibly induce expression of an ecdysone-responsive lacZ reporter gene following transient transfection. We simultaneously established stable Cos, Swiss 3T3, and human neuroblastoma EcR cell lines in an effort to generate an alternative assay system in which potential anti-proliferative effects of tightly controlled exogenous neurofibromin overexpression could be assessed. Similar analysis of reporter gene expression also indicated that no candidate line significantly induced expression of the lacZ reporter gene following treatment with the ecdysone analog. We then used an alternative method for generating cell lines in which we could tightly control exogenous neurofibromin expression. Dr. Xandra Breakefield and colleagues in the Molecular Neurogenetics Unit have considerable expertise in the development and use of amplicon vectors that permit reproducible and regulatable transgene expression in a wide variety of cell lines. Our initial studies utilized the HET2 amplicon vector shown in Figure 1A.

A



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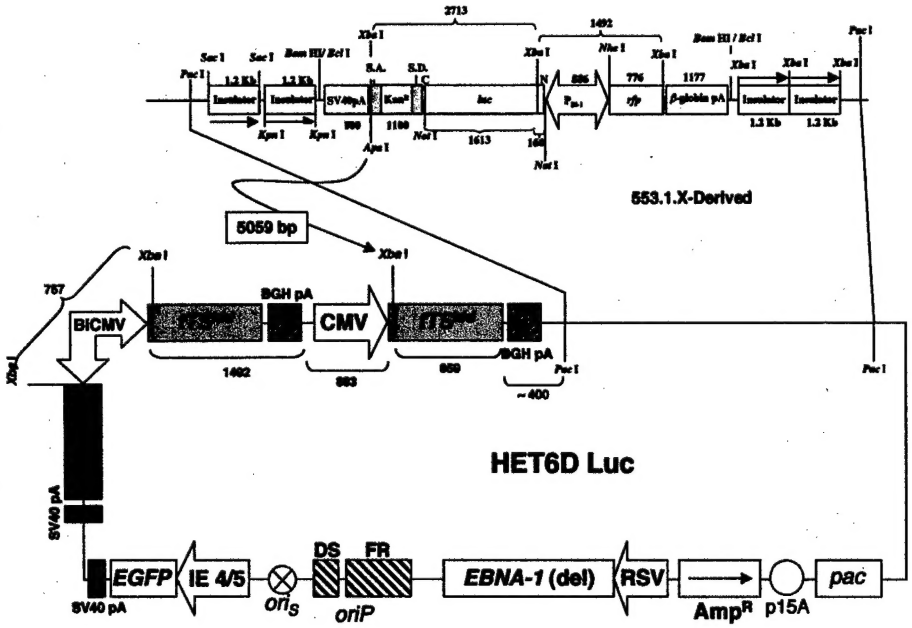

Figure 1 Tetracycline-regulated amplicons

The tetracycline-regulated amplicons HET2 (panel A) and HET6D Luc (panel B) are shown. The HSV/EBV amplicon backbone was derived from previously described constructs (9) and includes the reporter gene enhanced green fluorescent protein (EGFP, Clontech) under an immediate early viral promoter (HSV IE4/5), and the amplicon elements oriP, a latent origin of DNA replication, and a mutant version of the EBNA-1 gene. The amplicons also contain a cassette carrying the tet-silencer protein and the tet-activator protein under control of a bidirectional CMV promoter (10). In HET2, another cassette contains a multicloning site (MCS) for insertion of the gene of interest, and the reporter gene red fluorescent protein (RFP, Clontech) both of which are under the control of a bi-directional tet-responsive promoter (Clontech). HET6D Luc is derived from the HET2 vector and contains many of the same features of the amplicon backbone. The expanded region shows the elements surrounding the cloning site in which the transgene of interest replaces the luciferase coding sequence. The bidirectional promoter driving RFP and transgene expression is tetracycline-responsive.

The amplicon vectors have proven useful in the introduction and stable expression of genes in cells that are not readily transfected through conventional methods. The amplicon vector system is designed to produce high titer helper-free virus stocks that infect target cells with high efficiency (11). The vectors shown in Figure 1 allow transgene expression from a tetracycline regulatable promoter that also directs expression of a reporter (red fluorescent protein). These tetracycline-regulated amplicons are capable of high level dose-dependent induction of transgene expression in response to the application of the tetracycline analog doxycycline(M. Sena-Esteves and X. Breakefield, pers. comm.). The amplicons shown in Figure 1 also contain a tetracycline-independent reporter (enhanced green fluorescent protein) to monitor infection efficiency.

Our initial results using the HET2 amplicon (Figure 1A) were outlined in our midterm report. Briefly, we infected NIH3T3 cells with the amplicon carrying no transgene and examined both GFP and RFP expression in the absence of doxycycline 48 hours post-infection. We could readily detect GFP+ NIH3T3 cells indicating efficient amplicon infection. Although we could detect some GFP+/RFP- cells, we observed strong expression of the RFP reporter protein in the absence of doxycycline suggesting a high degree of constitutive transcription from the tetracycline-dependent promoter. Because low (2-3 fold) levels of neurofibromin overexpression in NIH3T3 cells inhibit proliferation, our studies require tight regulation of exogenous neurofibromin expression. This requirement prevented the use of the HET2 amplicon system in our studies and led us to again consider alternative expression systems.

In the past year we have focused on a retroviral version of the ecdysone-inducible mammalian expression system developed by Stratagene (12). One major advantage of this retroviral system is the availability of well-characterized stable NIH3T3 cell lines which express the ecdysone-responsive trans-activator component proteins at optimal levels for efficient and reproducible induction of introduced transgenes (13). Our major goals of the past year were the introduction of wild type and mutant neurofibromin proteins into the established NIH3T3 cell line available from Stratagene and subsequent analyses of the proliferation effects of each following controlled exogenous neurofibromin expression. Our progress has been severely limited by difficulties in generating a full-length neurofibromin cDNA clone in the ecdysone-responsive expression vector due to well-recognized toxic effects of neurofibromin expression in E. coli. We have utilized a variety of cloning strategies designed to overcome these problems but have not yet generated the relevant construct. This long-standing difficulty in manipulating the full-length neurofibromin cDNA clone makes the generation of multiple neurofibromin vectors designed for specific experimental purposes a daunting prospect. We are interested in developing neurofibromin expression vectors that could be used in studies outside the scope of this project. This approach requires an exogenous expression system that would not be specific to any particular cell type, allowing us to construct a single vector carrying the full-length neurofibromin cDNA that could then be used in multiple studies. The HET2 amplicon system was a good candidate due to the broad host range of the amplicon and we were disappointed in its high level of unregulated transgene expression. Recently the Breakefield laboratory has developed an improved version of the original HET2 vector which contains elements that are designed to reduce constitutive transcription from the bi-directional tetracycline-responsive promoter that drives transgene expression. This amplicon is shown in Figure 1B.

The major modification in the HET6D Luc amplicon is the presence of two copies of the *tTS*^{kid} gene which encodes a chimeric silencer protein that binds to the inducible promoter in the absence of tetracycline and blocks expression. Preliminary analysis of this amplicon indicates that the level of constitutive expression from the tetracycline-inducible promoter is significantly reduced as compared to the levels observed with the original HET2 vector (M. Sena-Esteves, pers. comm.). We have obtained

the HET6D Luc amplicon and have infected NIH3T3 cells to test the level of constitutive reporter gene expression from the tetracycline-regulated promoter. The results are shown in Figure 2.

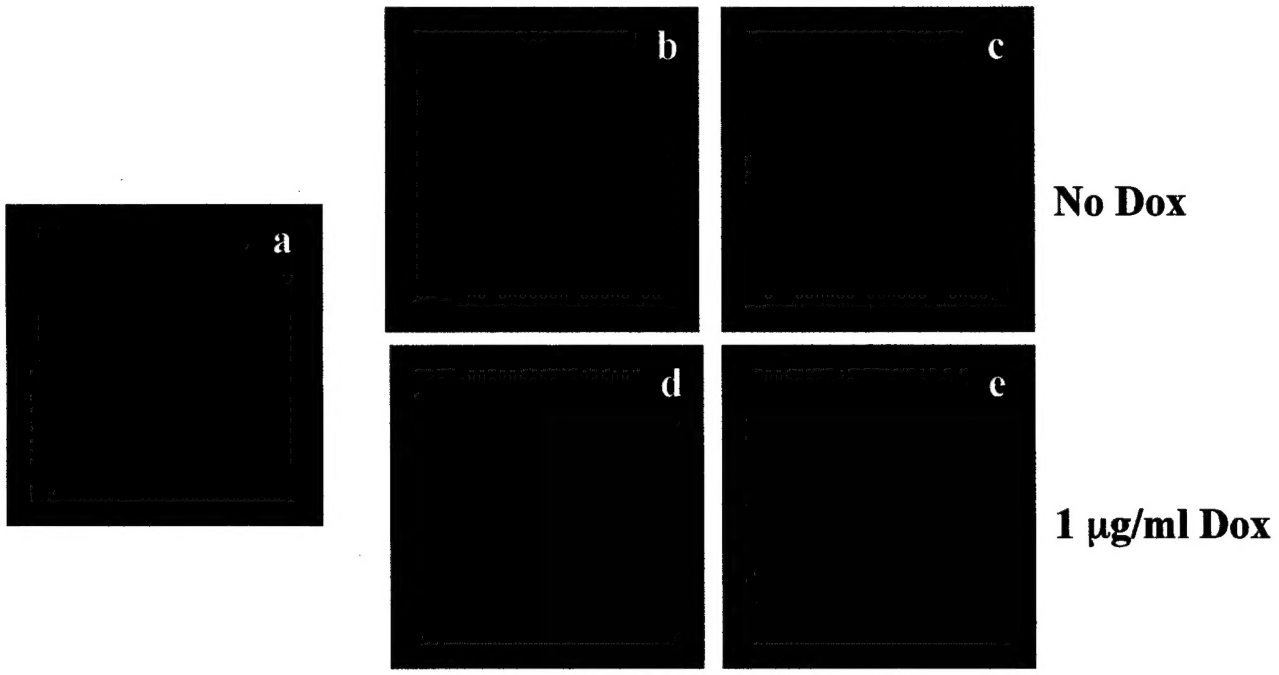

Figure 2 HET6D Luc infection of NIH3T3 cells and test of constitutive reporter gene expression
NIH3T3 cells were infected with the HET6D Luc amplicon at an MOI of 0.5. Expression of green fluorescent protein (GFP) was assessed by fluorescence microscopy 24 hours post-infection (panel a). Doxycycline was added to the cultures 24 hours post-infection and the levels of GFP and red fluorescent protein (RFP) expression were monitored at 72 hours after doxycycline addition. Panel b, GFP in the absence of doxycycline; panel c, RFP in the absence of doxycycline; panel d, GFP in the presence of 1 μg/ml doxycycline; panel e, RFP in the presence of 1 μg/ml doxycycline.

The NIH3T3 cells were readily infected by the Het6D Luc amplicon although the overall infection efficiency was low (panel a). We monitored both constitutive and inducible expression in the HET6D Luc-infected NIH3T3 cells by assessing RFP expression in the absence or presence of 1 μg/ml doxycycline at 72 hours following addition of the inducer. In the absence of doxycycline we saw no significant RFP expression in any GFP+ cells (compare panels b and c) indicating that there is little constitutive expression from the tetracycline-regulated promoter. Induction of RFP expression in the presence of doxycycline was detectable at 72 hours although only a subset of the GFP+ cells had significant levels of induced RFP expression (compare panels d and e). These preliminary results in the NIH3T3 cells are significant since it appears that there is improved control of constitutive expression in the HET6D Luc system compared to the HET2 amplicon. We are currently testing various infection and induction conditions to optimize transgene expression in the NIH3T3 cells. The HET6D Luc amplicon will be a valuable reagent in future experiments.

The broad host range of the amplicon vector system allows us to generate a single construct that can be used for analysis in both mouse and human cells relevant to other ongoing studies. We have tested the HET6D amplicon in human Schwann cell lines derived from neurofibromas associated with NF1. Recent progress in the isolation and culturing of human Schwann cells from neurofibromas indicates that these tumors contain genetically distinct populations of Schwann cells (14). Selective culture conditions allow the separate expansion of heterozygous *NF1+/-* cells and homozygous *NF1-/-* cells although the relative proportion of each subtype within the tumor has not been determined. We

have generated such cell lines from NF1-associated neurofibromas in collaboration with Dr. James Gusella in the Molecular Neurogenetics Unit. The Schwann cells used in our experiments were derived from two separate neurofibromas (T236 and T237) from a single individual. Schwann cell lines 236C and 237C were cultured under conditions that promote expansion of heterozygous *NF1* +/- cells. The cell lines 236F and 237F were cultured in the absence of forskolin which results in selective growth of *NF1* -/- Schwann cells (14). Figure 3 shows the infection of each of these lines with the HET6D Luc amplicon.

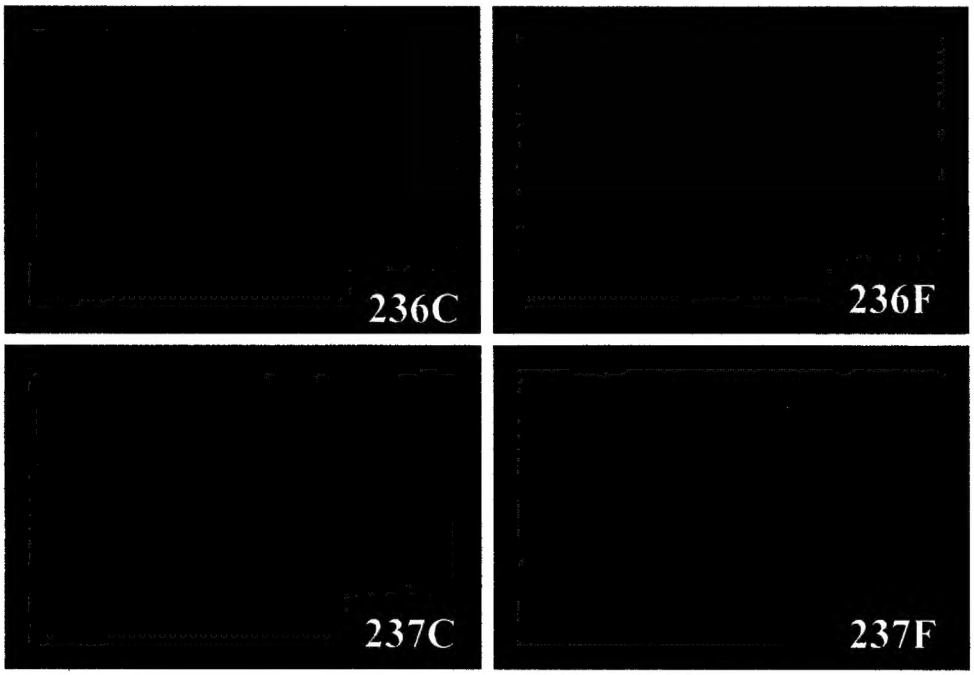

Figure 3 Infection of human Schwann cell lines with the HET6D Luc amplicon
Four separate human Schwann cell lines derived from NF1-associated neurofibromas were infected with the HET6D Luc amplicon at an MOI of 0.5. Expression of green fluorescent protein was assessed by fluorescence microscopy 24 hours post-infection.

We observed GFP+ cells in all tested Schwann cell lines although the overall infection efficiency was low. We monitored both constitutive and inducible expression in the HET6D Luc-infected 237C and 237F Schwann cells by assessing RFP expression in the absence or presence of 1 μg/ml doxycycline at 72 hours following addition of the inducer. The results are shown in Figure 4 (see next page). In the absence of doxycycline we saw no significant RFP expression in any GFP+ cells in both 237C and 237F indicating that there is little constitutive expression from the tetracycline-regulated promoter. We detected doxycycline-induced RFP expression in HET6D Luc infected 237C and 237F though only a subset of the GFP+ cells had significant levels of induced RFP expression. We obtained similar results with the 236C and 236F Schwann cell lines (data not shown). Our results with the HET6D Luc amplicon in the Schwann cell lines are significant because we can now use the amplicon to exogenously express wild-type and mutant neurofibromin in tumor derived cells. In this manner, we can study neurofibromin function in a pathologically relevant cell type.

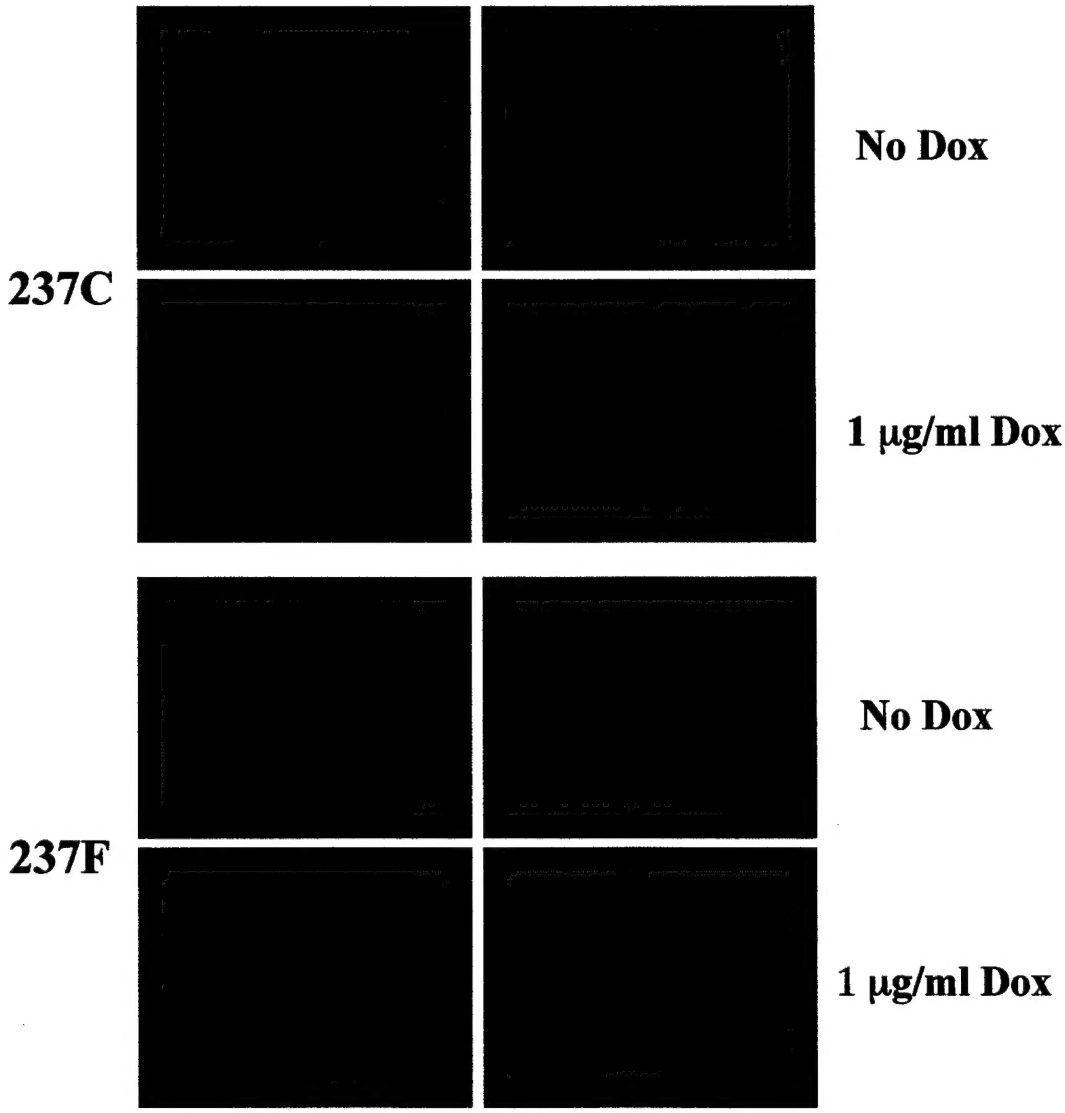

Figure 4 Induction of reporter gene expression in Het6D Luc-infected Schwann cells
The Schwann cell lines 237C and 237F were infected with the HET6D Luc amplicon at an MOI of 0.5. Doxycycline was added to the cultures 24 hours post-infection and the levels of GFP and RFP expression were assessed by fluorescence microscopy 72 hours after doxycycline addition.

Task 2. Construction and expression of mutant forms of neurofibromin

The goal of Task 2 was to generate mutant forms of neurofibromin in the ecdysone-inducible expression vector and test the ability of each to block NIH3T3 cell proliferation as a means to defining critical neurofibromin functional domains that are distinct from the GRD. The initial collection of mutants outlined in the grant proposal included single amino acid substitutions in the GRD that were predicted to eliminate interaction of neurofibromin with the Ras pathway. Comprehensive mutational analysis of the entire NF1 gene in a large cohort of affected patients has suggested that the region immediately amino-terminal to the GRD has a relatively high concentration of point mutations and may represent a distinct functional domain of neurofibromin. This region of homology is conserved in the Drosophila protein (4) and the yeast Ira1 and Ira2 proteins (14). We planned to construct and analyze amino acid substitution mutants in exons 12a (I581T, K583R), 15 (W777S, T780K, H781P), and 16 (L847P) in the proliferation assay since those exons are suggested to be critical regions within the proposed functional domain (8). We also planned to generate small in-frame deletion mutants in this

domain by use of convenient restriction sites or PCR-based mutagenesis. Our progress in the construction and analysis of these mutants was hampered by our difficulties in developing a reliable inducible expression system in NIH3T3 cells. We are currently generating a HET6D Luc-derived amplicon vector carrying FLAG-tagged wild-type neurofibromin. Similar constructs harboring epitope-tagged mutant forms of neurofibromin can also be generated and used in future studies of neurofibromin function.

Task 3. Generation of stable primary mouse embryo fibroblast/EcR cell lines

The goal of Task 3 was the generation of stable isogenic neurofibromin–deficient and wild type primary mouse embryo fibroblasts (MEFs) that express the ecdysone-responsive transcriptional activator that can be utilized in subsequent analyses of cellular proliferation following exogenous neurofibromin expression in the primary fibroblasts. We were interested in these analyses since the MEFs, unlike the pre-neoplastic NIH3T3 line, are not likely to have suffered any additional genetic changes and may have a different response to neurofibromin overexpression. As outlined in the midterm report, our progress in generating these lines using the Invitrogen system was limited by the low transfection efficiency of these cells and low cell viability following drug selection. Those difficulties were overcome by use of the HET2 amplicon system but the poor control of tetracycline-dependent expression in that system precluded its use in the MEF cell lines. Our preliminary results with the HET6D Luc amplicon in NIH3T3 cells were encouraging since this modified amplicon showed relatively tight control of constitutive transcription from the tetracycline-responsive promoter. We tested its utility in MEFs by simple infection of the neurofibromin-deficient MEF cell line 4528E. The results are shown in Figure 5.

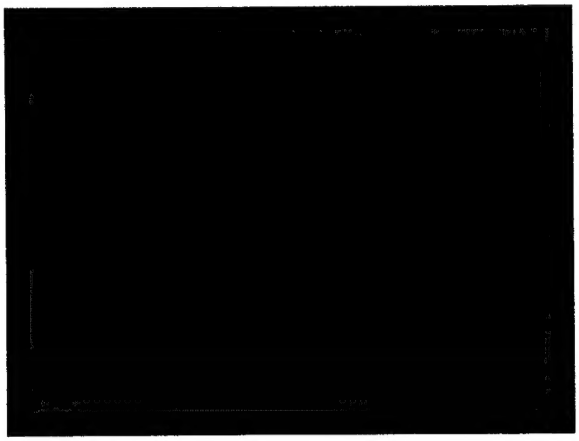

Figure 5 Infection of neurofibromin-deficient MEFs with the HET6D Luc amplicon
The neurofibromin-deficient mouse embryo fibroblast cell line 4528E was infected with the HET6D Luc amplicon at an MOI of 0.5. Expression of green fluorescent protein was assessed by fluorescence microscopy 24 hours post-infection.

The HET6D Luc amplicon infected the 4528E MEF cell line with reasonable efficiency. We tested infected cells for induction of the RFP reporter gene using the method outlined for the NIH3T3 fibroblasts and human Schwann cell lines. Our preliminary experiments were inconclusive due to decreased viability of the HET6D Luc-infected MEFs over the time course of the experiment. We are currently optimizing conditions for infection and induction in the neurofibromin-deficient MEFs.

Key Research Accomplishments N/A

Reportable Outcomes N/A

Conclusions

Our efforts over the entire funding period have been directed at surmounting one major obstacle to progress in NF1 research, namely the inability to manipulate normal and mutant neurofibromin expression in a relevant cell system. The development of reliable inducible expression in NIH3T3 cells using vector-and retroviral-based ecdysone controlled expression systems and the HET2 tetracycline-inducible amplicon system (task 1) was difficult and limited our ability to define critical functional domains of neurofibromin through analysis of mutant proteins (task 2). These limitations may be overcome by the use of the recently developed HET6D Luc amplicon system. Our recent work shows that the HET6D Luc amplicon affords tight regulation of exogenous gene expression in NIH3T3 cells and would be suitable for the proliferation studies outlined in our original grant proposal. Additionally, preliminary data indicate that the HET6D Luc amplicon system would allow analysis of potential anti-proliferation effects of exogenous neurofibromin expression in neurofibromin-deficient primary mouse embryo fibroblasts (task 3). Our analyses of tetracycline-regulated reporter gene expression in HET6D Luc-infected human Schwann cells derived from NF1-associated tumors showed tight control of expression. These results suggest that this amplicon system will be a valuable tool in analysis of neurofibromin function in a broad spectrum of cell types, including those that are pathologically relevant to the NF1 disease phenotype. We expect that further studies utilizing the HET6D Luc amplicon system will help clarify the complex role of neurofibromin in cellular growth control and may suggest additional cellular pathways distinct from Ras signaling in which the consequences of disease causing mutations in the *NF1* gene can be investigated. Moreover, these studies will provide insight into the molecular pathology of NF1 and identify potential targets for novel therapeutic approaches and management of this disorder.

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Appendices

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